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Development, physical stability and bioaccessibility of β-carotene-enriched tertiary emulsions

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ABSTRACT

The aim of this study was to develop a β-carotene-enriched tertiary emulsion (lactoferrin/alginate/ε-poly-L-lysine). Then, its physical stability as well as β-carotene content under external stresses (temperature, pH and ionic strength changes) was evaluated. Furthermore, lipid digestibility and β-carotene bioaccessibility of primary, secondary and tertiary emulsions were determined. Tertiary emulsion underwent a substantial particle size increase up to 13 μm after extreme temperatures, acidic conditions and with salt addition. However, small particle size (0.41 μm) and negative ζ-potential (−43.4 mV) was observed at basic pH. In addition, β-carotene content in tertiary emulsions decreased only 40% when emulsions were subjected at temperatures ≤70 °C, in acidic conditions and below 0.3 M NaCl. After *in vitro* digestion, tertiary emulsion presented higher lipid digestibility (83.59 ± 11.81%) and β-carotene bioaccessibility (70.10 ± 5.26%) compared with primary and secondary emulsions. This study provides knowledge to understand the behaviour of β-carotene-loaded tertiary emulsions under different conditions, valuable for further application in foodstuffs.

1. Introduction

Among carotenoids, β-carotene is one of the most extensively studied because of its health-related benefits, including anti-inflammatory (Li, Hong, & Zheng, 2019) and antioxidant activities (Zhou et al., 2018). Nonetheless, it has been reported that β-carotene is degraded by several factors including light, oxygen and heat, among others (Boon, McClements, Weiss, & Decker, 2010). Furthermore, β-carotene incorporation to aqueous-based foods is hindered because of its low-water solubility. Several techniques have been used to overcome the solubility issue, including lipid-based nanocarriers (nanoemulsions, nano-structured phospholipid carriers), nature-inspired nanocarriers (caseins, cyclodextrins), special equipment-based nanocarriers (electrospinning, electrospraying), biopolymer particles (single or complexed biopolymer nanoparticles) and miscellaneous nanocarriers (nanoparticles made from chemical polymers, nano-structured surfactants) (Assadpour & Mahdi Jafari, 2018). While a professional equipment is needed to produce the majority of these nanocarriers, a common and more available equipment can be used for producing nanoemulsions. Indeed, nanoemulsions have been proposed as a promising solution although they are highly reactive to external stimulations because of their nanometric particle size. Alternatively, multi-layer emulsions are oil-in-water emulsions containing oil droplets coated by at least two layers of polyelectrolytes. Because of the numerous layers lying around the oil droplets, multi-layer emulsions present improved physical stability over a wide range of conditions, including extreme pH, high ionic strength and thermal processing (Guzey & McClements, 2006). At the same time, the thick layer covering the oil droplets might provide chemical stability to encapsulated lipophilic compounds (Xu, Aihemaiti, Cao, Teng, & Li, 2016), as well as decrease lipid oxidation rates (Katsuda, McClements, Miglioranza, & Decker, 2008).

The assembly of layers is carried out following the layer-by-layer technique (LbL), in which polyelectrolytes with positive or negative charge are alternately deposited around the oil droplets (Bortnowska, 2015). Multi-layer emulsions fabrication is a delicate procedure as instability processes (e.g. bridging flocculation or depletion) could occur when an excess or lack of polyelectrolyte molecules is present (Guzey & McClements, 2006).

Literature reports multi-layer emulsions prepared with oil concentrations ranging from 0.09 to 2% w/w (Acevedo-Fani, Silva, Soliva-Fortuny, Martín-Belloso, & Vicente, 2017; Tokle, Mao, & McClements, 2013). Indeed, oil concentration is one critical point when designing

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multi-layer emulsions, since each layer deposition involves the dilution of the sample. When these systems are envisaged for delivering lipophilic bioactive compounds, the low oil concentration will allow incorporating only small amounts of bioactive compound within oil droplets. From a technical point of view, working with low oil concentrations in multi-layer emulsions makes difficult to assess their potential bioaccessibility after simulated *in vitro* digestions. Besides that, there is a controversy over whether the number layers covering multi-layer emulsions have an impact on lipolysis during *in vitro* digestion, thereby decreasing (Silva et al., 2018) or increasing (Silva et al., 2019) bioaccessibility of lipophilic compounds.

In terms of applicability, multi-layer emulsions can be employed as functional ingredients to improve nutritional quality of foods and beverages. However, processing and manufacturing conditions, as well as food characteristics might affect multi-layer emulsions properties. Thus, investigating how different external conditions affects physical stability of these emulsions would provide information about in which type of foodstuffs would be better to use multi-layer emulsions.

Previous studies have demonstrated that coated emulsions with at least two layers of polyelectrolytes are physically more stable under external conditions than uncoated emulsions (Zhang et al., 2015). Thus, the main purpose of this work was to develop β-carotene-enriched tertiary emulsions (lactoferrin-alginate-ε-poly-L-lysine) with a high oil concentration (2.5% w/w). Then, the effect of external stressing conditions (temperature, pH, and ionic strength changes) on the physical stability as well as on the β-carotene content of the tertiary emulsion was investigated. In addition, lipid digestibility and β-carotene bioaccessibility of the primary, secondary and tertiary emulsions after an *in vitro* digestion were evaluated.

2. Material and methods

2.1. Materials

Lactoferrin was kindly donated by Friesland Campina DOMO (Wageningen, The Netherlands). Sodium alginate (MW ≈ 26,000 g/mol, 60–65% Manuronic acid, 35–40% Guluronic acid) was provided by IMCD España Especialidades Químicas, S.A. (Barcelona, Spain). The ε-poly-L-lysine (Epolyly®, MW 30 kDa) was from Handary S.A (Brussels, Belgium). Corn oil (Koipe Asua) was purchased from a local market. Pepsin from porcine gastric (EC 3.4.23.1), pancreatin from porcine pancreatin (EC 232.468.9), sodium phosphate monobasic and β-carotene (synthetic, ≥93% (UV), powder) were from Sigma Aldrich. Magnesium chloride hexahydrate and potassium phosphate monobasic were from Acros Organics. Hydrochloric acid (HCl) and sodium chloride (NaCl) were from Poch S.A. Bile (EC 232.369.0), sodium azide, calcium chloride dehydrate were obtained from Fisher scientific. Sodium hydroxide (0.25 N) and potassium chloride were from Panreac. Milli-Q water was used to prepare all emulsions.

2.2. Methods

2.2.1. Preparation of emulsions

The polyelectrolytes combination for developing primary (lactoferrin), secondary (lactoferrin-alginate) and tertiary (lactoferrin-alginate-ε-poly-L-lysine) emulsions were selected based on a previous work carried out in our group (Acevedo-Fani et al., 2017). However, a suitable polyelectrolyte concentration for each interfacial layer deposition was optimized since the β-carotene emulsions contained a higher oil concentration, compared to our previous study.

First, the lipid phase containing 0.5% w/w β-carotene was prepared by mixing the bioactive compound with corn oil through sonicating (1 min) (Hielscher Ultrasound Technology, Teltow, Germany, UP-400S) and stirring (5 min, 45 °C). To prepare the primary emulsions, the lipid phase (20% w/w) and the aqueous phase (80% w/w) with different lactoferrin concentrations (2.5–7.5% w/w), were mixed with an ultraturrax (Janke & Kunkel, Staufen, Germany) at 5000 rpm for 3 min. After that, this coarse emulsion was passed through a Microfluidizer (Microfluidics M−110P) at a pressure of 100 MPa for 5 cycles. The term primary emulsion was referred to the emulsion containing oil droplets coated by lactoferrin. The optimal formulation selected was used to formulate secondary emulsions.

To prepare secondary emulsions, 10 mL of primary emulsion were added to 10 mL of alginate solutions under continuous agitation (200 rpm) at a dripping rate of 10 mL/min. Afterwards, secondary emulsions were stirred (500 rpm) for 15 min in order to disrupt any flocs formed. The term secondary emulsions was referred to emulsions containing oil droplets coated with two layers (lactoferrin-alginate). The resulting secondary emulsion contained 0.25% (w/w) β-carotene, 10% (w/w) corn oil, 2.5% (w/w) lactoferrin and different concentrations of alginate (between 0.8% w/w and 1.2% w/w). The optimal formulation selected after analysing the data was used to formulate tertiary emulsions.

To prepare tertiary emulsions, 5 mL of secondary emulsion were added to 15 mL of ε-poly-L-lysine solutions under continuous stirring (200 rpm), at a dripping rate of 10 mL/min. Then, tertiary emulsions were stirred (500 rpm) for 15 min and then sonicated during 2 min (cycle 0.5, 40 Hz) (Hielscher Ultrasound Technology, Teltow, Germany, UP-400S). This helped to disrupt flocs formed during the process. The term tertiary emulsion was referred to emulsions containing oil droplets coated by three layers (lactoferrin-alginate-ε-poly-L-lysine). The resulting tertiary emulsion contained 0.0625% (w/w) β-carotene, 2.5% (w/w) corn oil, 0.62% (w/w) lactoferrin, 0.25% (w/w) alginate and different concentrations of ε-poly-L-lysine, ranging from 0 to 0.25% (w/w).

The pH of all polyelectrolyte solutions and emulsions was 5. This is the pH at which polyelectrolytes are strongly oppositely charged and can form electrostatic complexes around oil droplets.

2.2.2. Optimization of polyelectrolytes concentration in emulsions

The most suitable polyelectrolyte concentration to create primary, secondary and tertiary emulsions was determined by measuring different parameters. For primary emulsions, protein surface load, particle size and surface charge were measured, whereas for secondary and tertiary emulsions, particle size and surface charge were determined. Furthermore, optical images were used to observe destabilisation processes during the interfacial layer's assembly.

To have more information about optimized primary, secondary and tertiary emulsions, their encapsulation efficiency (Davidov-Pardo & McClements, 2015) as well as span index (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK) (Velderrain-Rodríguez, Acevedo-Fani, González-Aguilar, & Martín-Belloso, 2019) were determined. In addition, TEM images were used to provide information about emulsions' structure and morphology.

2.2.2.1. Protein surface load determination. Protein surface load was determined in primary emulsions using a method adapted from Ye (2010). This analysis consists on determining the minimum concentration of polyelectrolyte (in this case lactoferrin) needed to create a coating on the oil droplets surface of a primary emulsions. Briefly, primary emulsions were centrifuged for 30 min, at 1000 rpm (AVANTI J-25, Beckman Instruments Inc., Fullerton, CA, USA) and the aqueous phase (bottom layer of the centrifuge tube) was separated from the lipid phase (top layer). This procedure was repeated by re-dispersing the lipid phase in Milli-Q water and centrifuging again. This was done to ensure all lactoferrin of aqueous phase was extracted. The aqueous phase obtained after the first and second centrifugation were joined and passed through a 0.22 μm filter. The protein concentration was measured by the Bradford method (Thermo Scientific Pierce Coomassie Protein Assay Kit), using lactoferrin as standard (Bradford, 1976). Then, protein surface load in emulsions was calculated following Eqs. (1) and (2):

$$C_{adsorbed} = C_{initial} - C_{aqueous\ phase} \quad (1)$$

where $C_{adsorbed}$ is the lactoferrin concentration adsorbed at the oil droplets surface, $C_{initial}$ is the lactoferrin concentration added to the emulsions and $C_{aqueous\ phase}$ is the lactoferrin concentration quantified in the aqueous phase.

Therefore, protein surface load (Γ), which indicated lactoferrin mass adsorbed at the droplets interface, was calculated as follows:

$$\Gamma = \frac{d_{32} \times C_{adsorbed}}{6 \times \Phi} \quad (2)$$

where ϕ is the oil volume, Γ is the surface load of lactoferrin (kg/m^2), d_{32} is the oil droplet diameter (meters) and $C_{adsorbed}$ is the amount of lactoferrin adsorbed at the oil droplet surface per emulsions volume (kg/m^3).

2.2.2.2. Physicochemical determination. Particle size was determined using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK), reporting results as the surface are mean diameter, $D_{[3,2]}$ (μm). The refractive index of the corn oil and water were 1.47 and 1.33, respectively.

Oil droplets surface charge (ζ-potential) was determined using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcerstershire, UK). Samples were previously diluted 1/10 with Milli-Q water (pH ≈ 6.5). Temperature was set to 25 °C for all analyses.

For optical microscopy images of emulsions, a drop of freshly prepared emulsion was placed on a glass slide and a cover slip on top. Images were taken using an optical microscope BX41 (Olympus, Shinjuku, Japan), linked to a digital camera Olympus DP74 (Olympus, Shinjuku, Japan) at a magnification of 100×. For TEM images, samples were prepared by depositing each emulsion on a carbon-coated copper grid, and negatively stained with 2% (w/v) uranyl acetate for observation. Samples were air-dried before observation with a transmission electron microscopy (TEM) (Jeol JEM-1010, Tokyo, Japan) working at 80 kV.

2.2.3. Tertiary emulsions stability to external stressing conditions

Tertiary emulsions were exposed to different external conditions such as temperature, pH and ionic strength changes. Temperatures ranged from 30 °C to 90 °C, emulsions were adjusted at different pH conditions from 2 to 11, and ionic strength was modified by adding different NaCl concentrations to emulsions (0–0.5 M).

Physical stability of tertiary emulsions under these conditions was assessed after 24 h exposure to these conditions as described in Section 2.2.2.2. Amount of β-carotene in tertiary emulsions was quantified spectrophotometrically (Ultrospec 3000 pro GE Health Sciences, USA) (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013) as well as presence of creaming was visually checked after 24 h of treatment.

2.2.4. Emulsions functionality

2.2.4.1. Lipid digestibility. An *in vitro* static model of digestion was used to subject primary, secondary and tertiary emulsions throughout gastric and intestinal conditions (Minekus et al., 2014). For the stomach phase, each emulsion was mixed with simulated gastric fluids (SGF) containing pepsin (2000 U/mL in the final mixture), $CaCl_2$ (H_20) (0.3 M), Milli-Q water and HCl (1 M) and incubated (Incubator OPAQ, OVAN, Barcelona, Spain) during 2 h at 37 °C with continuous agitation (100 rpm). Afterwards, the sample from the stomach phase was placed in a water bath (37 °C) to simulate the intestinal digestion and monitor lipolysis using a pH-stat (Metrohm USA Inc., Riverview, FL, USA). Simulated intestinal fluids (SIF) (0.150 M NaCl and 0.01 M $CaCl_2$), bile extract (54 mg/mL) and pancreatin (75 mg/mL) were added to the sample. During intestinal digestion, lipase hydrolyses ester bonds from lipid droplets (triacylglycerols) into several lipid digestion products, including diacylglycerols (DAG), monoacylglycerds (MAG), free fatty acids (FFA) and glycerols. The release of these FFA cause pH reduction in the samples and the constant tritation with 0.25 M NaOH maintain the pH at 7 during 2 h. The total volume of NaOH employed during intestinal digestion was used to calculate the lipid digestibility through total FFA release content, using Eq. (3).

$$FFA(\%) = \frac{V_{NaOH} \times C_{NaOH} \times M_{Oil}}{2 \times m_{oil}} \times 100 \quad (3)$$

where V_{NaOH} is NaOH volume (L) used to compensate the FFAs during the digestion, C_{NaOH} is NaOH molarity (0.25 mol/L), M_{oil} is corn oil molecular weight (800 g/mol), m_{oil} is corn oil total weight present in emulsions (g).

2.2.4.2. β-carotene bioaccessibility. Micellar fraction was obtained by centrifuging (AVANTI J-25, Beckman Instruments Inc., Fullerton, CA, USA) the liquid collected after the *in vitro* digestion at 4000 rpm for 40 min at 4 °C (Qian, Decker, Xiao, & McClements, 2012). The upper part of the centrifuged liquid was the micellar fraction, in which the mixed micelles formed during the *in vitro* digestion containing the solubilised β-carotene were suspended. In the centrifuged samples, a layer of oil could be observed on top of the liquid, which was dismissed since it was considered non-digested oil after *in vitro* digestion.

The concentration of β-carotene in undigested emulsions (initial) and micellar fraction was determined using a previously reported method (Salvia-Trujillo et al., 2013). Briefly, samples were mixed with chloroform, vortexed and centrifuged. This process was repeated until the upper part of the mixture became colourless. The bottom chloroform part containing the solubilised β-carotene was measured by UV–visible spectrophotometry (Ultrospec 3000 pro GE Health Sciences, USA) at 450 nm, using chloroform as a blank. Lastly, the β-carotene bioaccessibility was calculated using Eq. (4).

$$Bioaccessibility(\%) = \frac{C_{micelle}}{C_{intial}} \times 100 \quad (4)$$

where $C_{micelle}$ and $C_{initial}$ are the β-carotene concentration of the micellar fraction and the undigested emulsion, respectively.

2.2.5. Statistical analysis

The analysis of variance (ANOVA) was conducted using Statgraphics Plus v.5.1 Windows package (Statistical Graphics Co., Rockville, Md, USA) to identify samples with significant differences ($p \leq 0.05$ was considered significant). All parameters were performed in triplicate and data was reported as the mean with standard deviation.

3. Results and discussion

3.1. Primary emulsion formation: Effect of lactoferrin concentration

Primary emulsions presented particle sizes ($D_{3,2}$) below 0.41 μm and positive ζ-potential (between +51.9 mV and +55.3 mV) regardless the lactoferrin concentration. A significant reduction in particle size until 0.29 μm was observed when 5% (w/w) of lactoferrin was used to prepare the emulsion (Table 1). In fact, these results were in agreement

Table 1
Particle size ($D_{3,2}$(μm)), ζ-potential (mV) and surface load (mg/m^2) of primary emulsions containing different concentrations of lactoferrin (LF). Different letters mean significant differences ($p < 0.05$) between emulsions with different concentrations of lactoferrin.

LF (%)	$D_{3,2}$ (μm)	ζ-potential (mV)	Surface load (mg/m^2)
2.5	0.41 ± 0.02^{c}	52.85 ± 1.95^{a}	2.60 ± 0.46^{a}
4	0.39 ± 0.01^{bc}	52.13 ± 0.50^{a}	6.04 ± 0.04^{b}
4.5	0.39 ± 0.01^{bc}	53.15 ± 3.19^{a}	6.32 ± 0.01^{b}
5	0.29 ± 0.03^{a}	51.90 ± 2.08^{a}	10.22 ± 0.31^{cd}
6	0.37 ± 0.01^{b}	55.35 ± 1.15^{a}	9.91 ± 0.83^{c}
7.5	0.39 ± 0.02^{bc}	52.56 ± 5.75^{a}	10.94 ± 0.42^{d}

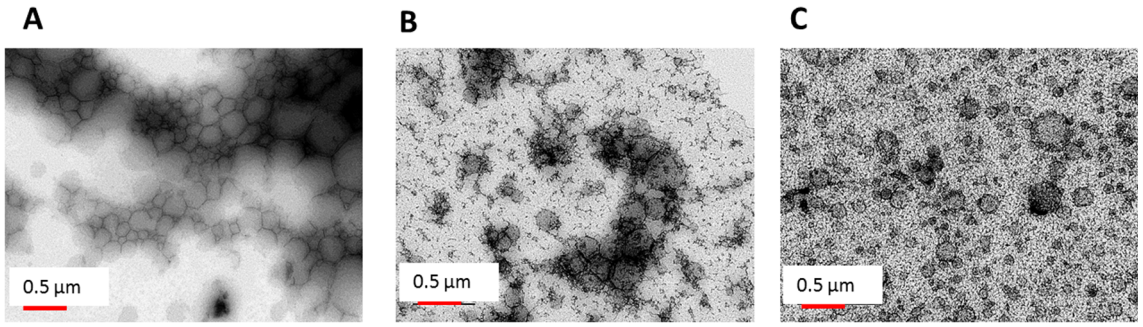

Fig. 1. Images of primary (A), secondary (B) and tertiary emulsion (C) obtained by Transmission Electron Microscopy (TEM). The magnification was 40.000x.

with surface load results, in which a maximum value of 10.22 mg/m^2 was reached at a lactoferrin concentration of 5% (w/w), indicating that there were enough lactoferrin molecules covering the oil droplets of primary emulsion, thereby preventing droplet aggregation (Mao & McClements, 2011). In addition, TEM images confirmed the formation of primary emulsions, which showed a spherical shape and a fine interface, corresponding to lactoferrin coating (Fig. 1A).

The high molecular weight of lactoferrin (around 80 kDa) together with its distant isoelectric point (≈8) from pH of emulsions (≈5), would have stabilised oil droplets by a combination of both steric and electrostatic forces (Tokle & McClements, 2011). At higher lactoferrin concentrations (> 5% w/w), emulsions underwent a significant increase of particle size, which may indicate that part of the extra lactoferrin added would have remained unabsorbed. This extra lactoferrin prevailed in the aqueous phase producing attractive forces between droplets, which led to droplet-droplet interactions (Israelachvili, 2011).

Therefore, the primary emulsion containing 5% w/w of lactoferrin was selected as the optimal formulation to proceed with the formation of the secondary emulsion.

3.2. Secondary emulsion formation: Effect of alginate concentration

A significant increase in particle size of secondary emulsions from 0.29 μm to 0.68 μm was observed when alginate was added. The mininum particle size of secondary emulsions was observed at 1% (w/w) alginate (0.45 μm) and adding concentrations of alginate above 1% (w/w) resulted in a significant increase of the particle size (Fig. 2A). Indeed, optical microscope images of secondary emulsions stabilised with low (0.8% w/w) and high (1.2% w/w) concentrations of alginate presented some droplet aggregation, while no destabilisation phenomena was observed in emulsions that contained alginate at 1% (w/w) (Fig. 2B). On one hand, when the polyelectrolyte content in the emulsion was insufficient to cover the entire droplet surface, a single molecule of polyelectrolyte would have been adsorbed at the surface of different droplets simultaneously, linking them together and resulting in large particle size (bridging flocculation) (Guzey & McClements, 2006). On the other hand, depletion floculation effect driven by an excess of polyelectrolyte would have been induced by the unadsorbed molecules surrounding the droplets, resulting in attractive forces and consequent droplet aggregation (Zeeb, Thongkaew, & Weiss, 2014).

Regarding the ζ-potential, adding alginate to primary emulsions resulted in a change of ζ-potential from positive (+42 mV) to negative values (−56 mV) (Fig. 2A). As alginate and lactoferrin have opposite charges (anionic and cationic, respectively), alginate would have been adsorbed to lactoferrin-coated oil droplets surface by electrostatic interactions, leading to the formation of a second interfacial layer.

Based on these results, the optimal alginate concentration for formulating the secondary emulsion was 1% (w/w), as it presented the lowest values of particle size (0.45 μm) and a strong negative ζ-potential (−55.20 mV). According to TEM images, secondary emulsion also presented spherical shape and a slightly dark line around the droplets, which was the lactoferrin-alginate coating (Fig. 1B).

3.3. Tertiary emulsion formation: Effect of ε-poly-L-lysine concentration

Tertiary emulsions with a low ε-poly-L-lysine concentration (0.12% and 0.15% w/w) presented particle sizes around 0.30 μm, whereas adding concentrations up to 0.17% w/w significantly increased the particle size of emulsions (≈1.85 μm). Furthermore, the initial ζ-potential of these secondary emulsions increased from −55 mV to −9.36 mV when tertiary emulsions contained up to 0.17% (w/w) of ε-poly-L-lysine (Fig. 3A). These results indicated that there were not enough ε-poly-L-lysine molecules covering the lactoferrin/alginate-coated droplets and that alginate was the polyelectrolyte prevalent at the droplets interface. When tertiary emulsions contained concentrations of ε-poly-L-lysine between 0.18% (w/w) and 0.19% (w/w) a significant reduction in particle size was observed (0.59 μm and 0.58 μm, respectively). Furthermore, these emulsions exhibited positive ζ-potential indicating that ε-poly-L-lysine had been deposited at the droplets surface. However, a steep increase in particle size until 14 μm and droplet aggregation (using optical microscope images) was detected at higher concentrations (0.20% and 0.25% w/w) (Fig. 3A and 3B). High ε-poly-L-lysine concentrations in tertiary emulsion would have favoured depletion flocculation and thus, droplet aggregation (Benjamin, Silcock, Leus, & Everett, 2012).

Taking into account all the results, two different concentrations of ε-poly-L-lysine (0.18% w/w and 0.19% w/w) could be selected as optimal concentration in the formation of the tertiary emulsion. However, tertiary emulsion containing 0.18 w/w of ε-poly-L-lysine presented high encapsulation efficiency and good span index indicating homogeneity (Table 2). TEM images confirmed the lactoferrin-alginate-ε-poly-L-lysine coating because of the intense black coverage around the droplets (Fig. 1C). Moreover, the lowest content of ε-poly-L-lysine in tertiary emulsions (0.18% w/w) could be related to economically and environmentally sustainable food systems. The following experiments of the study were carried out with the tertiary emulsion obtained after optimization process.

3.4. Tertiary emulsions stability to external stressing conditions

3.4.1. Temperature

Tertiary emulsions were stable at temperatures ≤ 60 °C since there were no changes in the particle size and ζ-potential as well as no evidence of droplet aggregation. On the other hand, tertiary emulsions presented a progressive particle size increase and some droplet aggregation at higher temperatures (70–90 °C) (Fig. 4B). At the same time, ζ-potential of these tertiary emulsions significantly decreased from + 13 mV to + 3.45 mV (Fig. 4A). Large particle sizes in emulsions heated above 60 °C could be related with a partial detachment of polyelectrolytes molecules from the droplets, thereby sharing one polyelectrolyte molecule with more than one droplet (bridging flocculation) (Guzey & McClements, 2006). Furthermore, proteins are heat

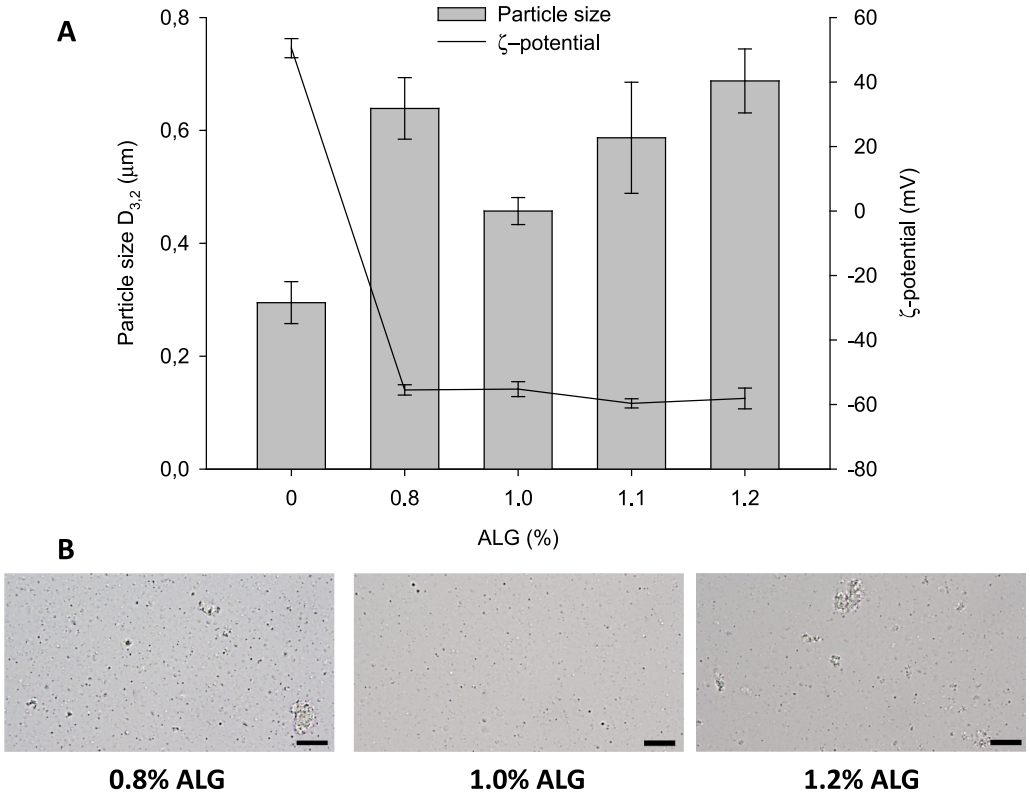

Fig. 2. (A) Particle size ($D_{3,2}$(μm)) and ζ-potential (mV) of secondary emulsions containing different concentrations of alginate (ALG). (B) Optical microscopy images of secondary emulsions. Scale bars were 10 μm long.

sensitive molecules, while polysaccharides such as alginate tend to be temperature independent (Dickinson, 2003). Thermal denaturation of lactoferrin (between 70 °C and 85 °C) could have derived into protein unfolding, thus promoting hydrophobic attraction between droplets (Teo, Lee, & Goh, 2017). In addition, high temperatures could have changed ε-poly-L-lysine structure, particularly to β structures (Mirtič &

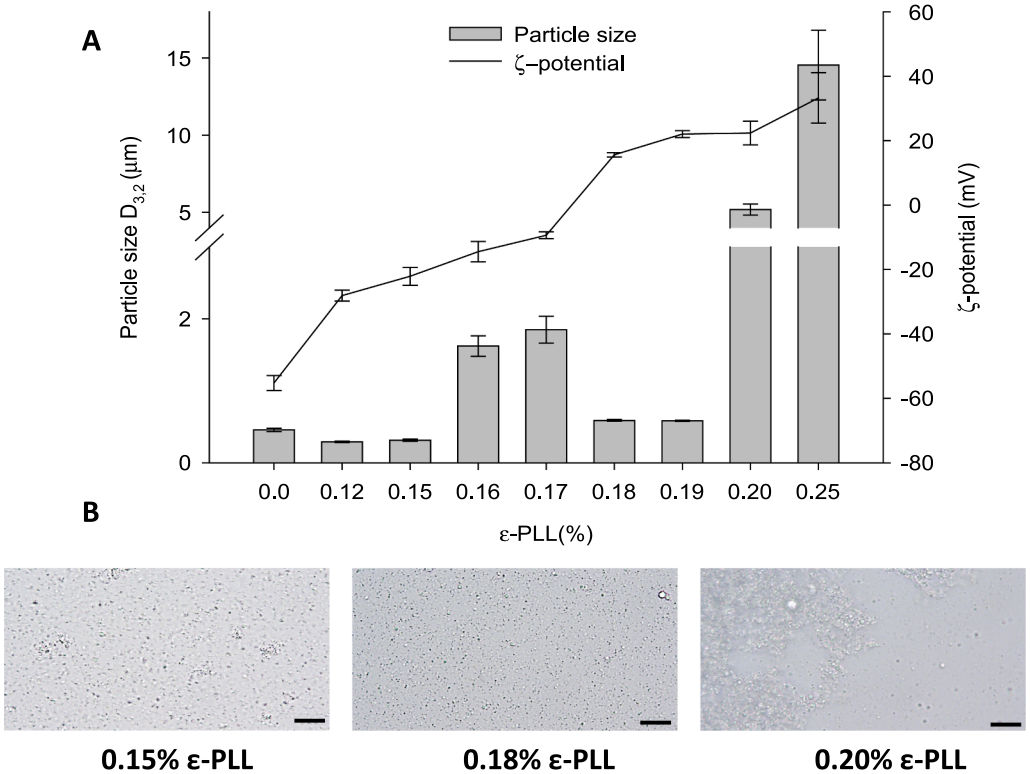

Fig. 3. (A) Particle size ($D_{3,2}$(μm)) and ζ-potential (mV) of tertiary emulsions containing different concentrations of ε-poly-L-lysine (ε-PLL). (B) Optical microscopy images of tertiary emulsions. Scale bars were 10 μm long.

Table 2
Encapsulation efficiency of β-carotene and span index for primary, secondary and tertiary emulsions. Different letters mean significant differences ($p < 0.05$) between emulsions with different interfacial layers.

Emulsions	β-carotene encapsulation efficiency (%)	Span index
Primary	82.52 ± 0.60[a]	1.32 ± 0.05[a]
Secondary	96.63 ± 1.50[b]	1.42 ± 0.05[b]
Tertiary	96.06 ± 0.35[b]	2.19 ± 0.15[c]

Grdadolnik, 2013) characterised to have a compact structure and thus, less ε-poly-L-lysine amine groups charged (Lee, 2016). These phenomena could explain the reduction of tertiary emulsion ζ-potential to nearly zero values, leading to poor electrostatic stabilisation and further droplet aggregation. However, no visual creaming in any of the tertiary emulsions was observed after 24 h treatment (Fig. 4A). The creaming process not only depends on the particle size, but also on the density difference between the continuous and dispersed phase. When the density of the dispersed phase (oil) is lower than the density of the continuous phase (water), oil droplets move upwards thereby creating a

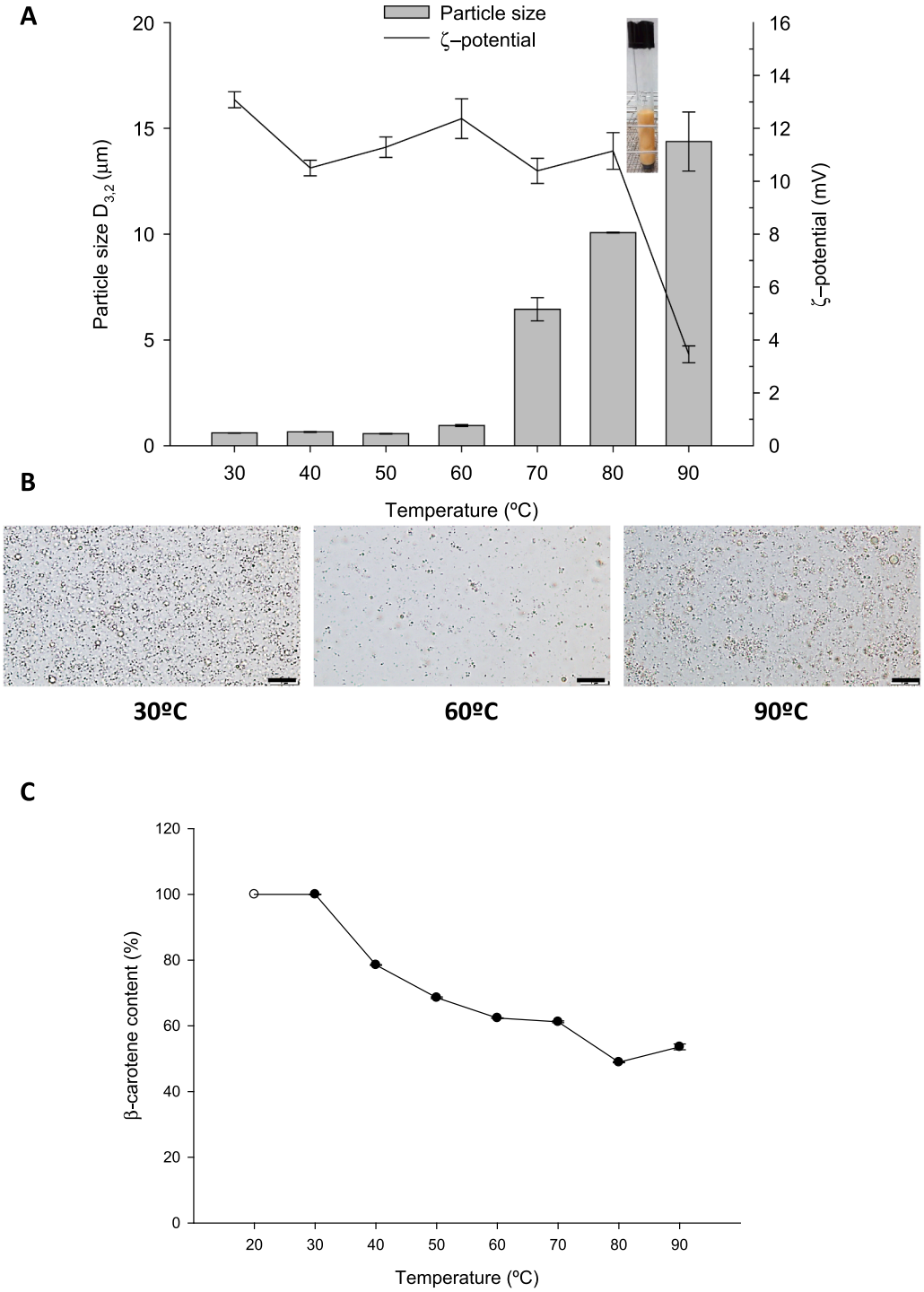

Fig. 4. (A) Influence of different temperatures on particle size ($D_{3,2}$(μm)) and ζ-potential (mV) of tertiary emulsions. (Inset) Visual image of tertiary emulsions after 24 h treatment. (B) Optical microscopy images of tertiary emulsions at different temperatures. Scale bars were 10 μm long. (C) Influence of different temperatures on β-carotene content (%) within tertiary emulsions.

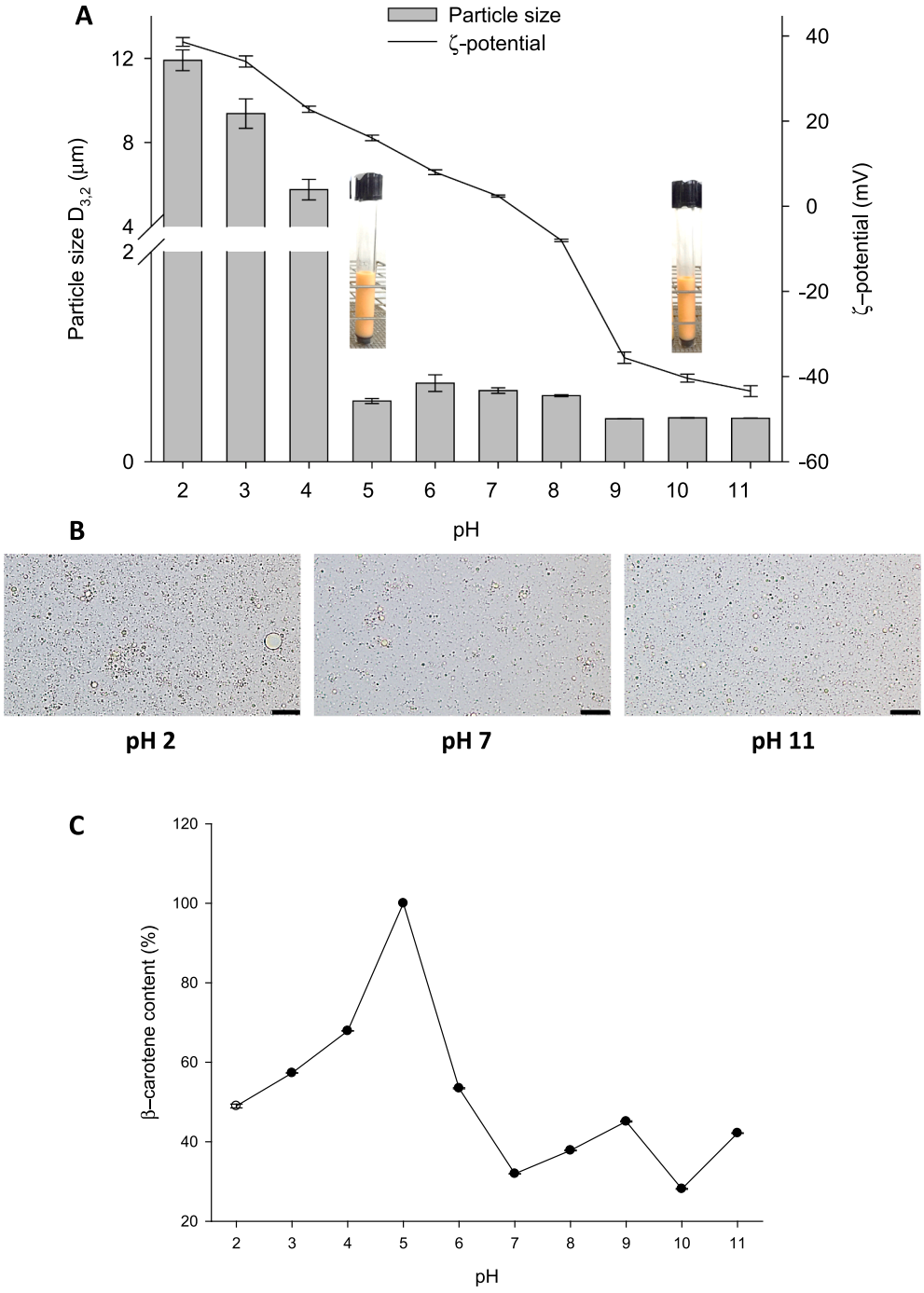

Fig. 5. (A) Influence of pH conditions on particle size ($D_{3,2}$(μm)) and ζ-potential (mV) of tertiary emulsions. (Insets) Visual images of tertiary emulsions after 24 h treatment. (B) Optical microscopy images of tertiary emulsions at different pH. Scale bars were 10 μm long. (C) Influence of different pH conditions on β-carotene content (%) within tertiary emulsions.

phase separation. In this study, tertiary emulsion could have retarded the creaming process because the layers covering oil droplets would have reduced the density contrast between dispersed and continuous phases, decreasing the gravitational separation of both phases (McClements, 2005). Because of its unsaturated structure, β-carotene is susceptible to degradation under high temperatures. Accordingly, β-carotene was constantly degraded at temperatures above 40 °C (Fig. 4C), although its content in tertiary emulsion remained always above 50%, irrespective of the temperature applied.

3.4.2. pH

The particle size of tertiary emulsions progressively increased from 0.57 μm to 11.91 μm as the pH of emulsions decreased from 5 to 2, while no significant changes in particle size were observed at higher pH values (from 6 to 11). However, none of the emulsions exhibited creaming after 24 h treatment. Regarding ζ-potential, tertiary emulsions presented a positive charge at pH below 5, being highly positive at pH 2 (+38 mV). On the other hand, when the pH of tertiary emulsions increased from 6 to 11, ζ-potential changed progressively from +15 mV to −43 mV. Specifically, emulsions presented ζ-potential close to 0 mV when the pH was between 7 and 8, and then it changed to negative when the pH of tertiary emulsions further increased (pH > 9) (Fig. 5A).

Under acidic environments (pH between 2 and 5), the pH of

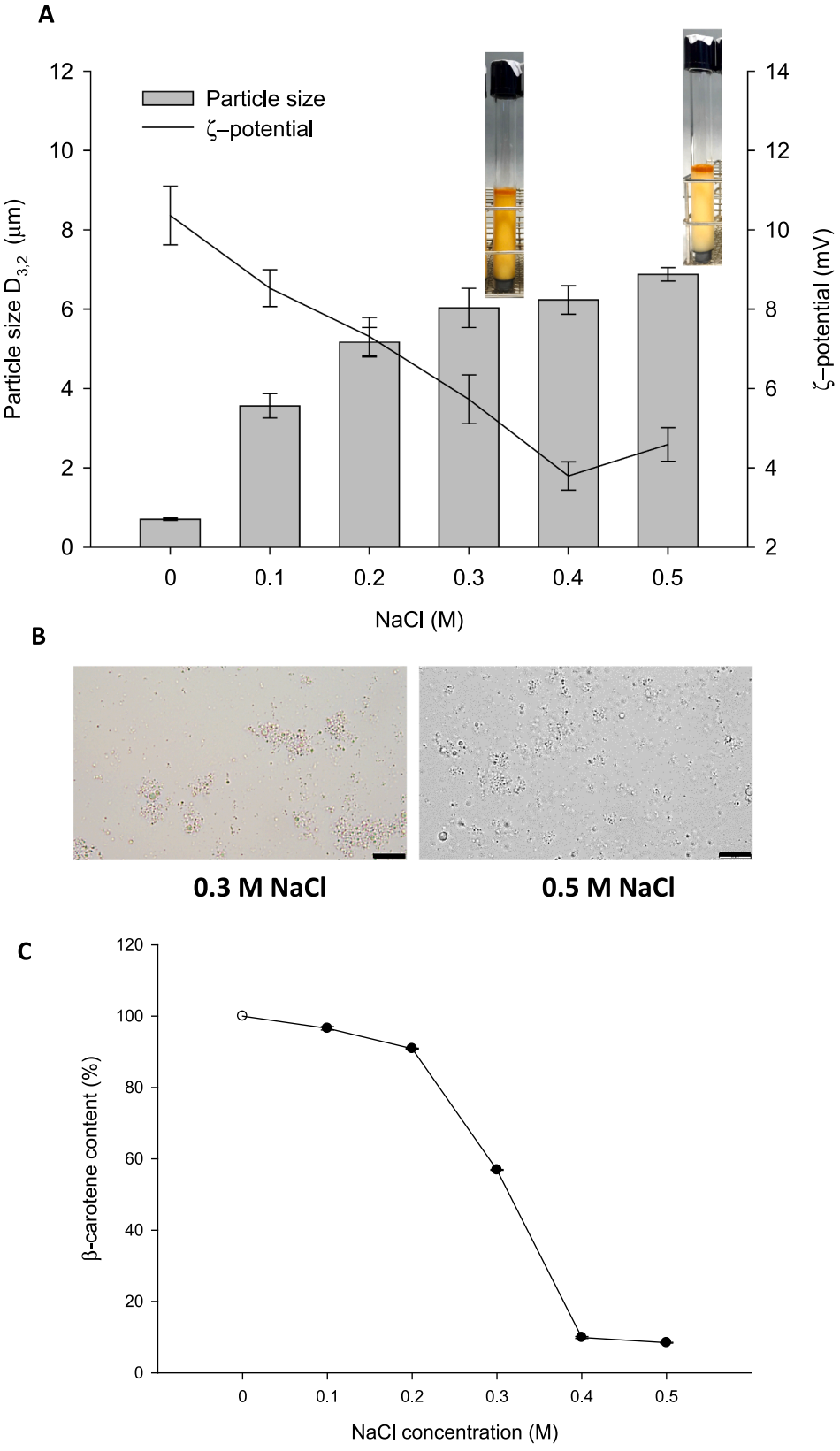

Fig. 6. (A) Influence of salt addition (NaCl) on particle size ($D_{3,2}$(μm)) and ζ-potential (mV) of tertiary emulsions. (Insets) Visual images of tertiary emulsions after 24 h treatment. (B) Optical microscopy images of tertiary emulsions at different NaCl concentrations. Scale bars were 10 μm long. (C) Influence of salt addition (NaCl) on β-carotene content (%) within tertiary emulsions.

emulsions was below the pK_a of ε-poly-L-lysine (≈9) and close to alginate pK_a (≈3.5) (Lee & Mooney, 2012). In this situation, electrostatic attraction between ε-poly-L-lysine (highly charged) and alginate (partially uncharged) would have been reduced, leading to a weak attachment between both components. These results suggested that the thickness of the interface was reduced, and ε-poly-L-lysine stabilisation

via steric repulsion was not enough to overcome attractive forces. Indeed, droplet aggregation could be observed in optical microscope images when emulsions had acidic pH (Fig. 5B).

When basic conditions were present (pH between 8 and 11), ε-poly-L-lysine lost its charge ($pK_a \approx 9$) and thus the electrostatic attraction with alginate might have been reduced, leading to a detachment of ε-poly-L-lysine from alginate-covered droplets. In this case, alginate was the polyelectrolyte that predominated at the interface of the droplets, contributing on the obtained negative ζ-potential. Either a particle size increase or droplet flocculation in tertiary emulsions were not observed in this situation (Fig. 5B), which might be attributed to the thick and charged layer of alginate covering the droplets, providing both steric and electrostatic stabilisation to the system (McClements, 2004). Under neutral pH conditions (pH between 6 and 7), tertiary emulsions presented very low or almost no surface charge and no droplet aggregation. These results suggested that the stability of these tertiary emulsions was not determined by charge (electrostatic repulsion), but by the polyelectrolyte coating around the oil droplets (steric repulsion).

In general, β-carotene content was reduced when pH changed from the original pH employed to form the tertiary emulsion (≈5) to either acidic (49% of β-carotene) or basic conditions (28% of β-carotene) (Fig. 5C). The interfacial layers of tertiary emulsion might act as a physical barrier to avoid the interaction between β-carotene and free radicals and/or oxygen, which would lead to compound degradation. Although tertiary emulsions were physical stable under basic environments, changes in the interfacial layer of the emulsion would have not been enough to prevent β-carotene degradation.

3.4.3. Ionic strength

With increasing NaCl concentration, particle size of tertiary emulsions was significantly incremented (≈7 μm), resulting in extensive droplet aggregation (Fig. 6A, 6B). Furthermore, ζ-potential exhibited a progressive decrease from +10 mV to +4.5 mV when NaCl concentration increased from 0 to 0.5 M (Fig. 6A). These results suggested that the presence of ions in the aqueous solution from NaCl (Cl^-) might have interacted with positively charged amino groups of ε-poly-L-lysine molecules, leading to either a partial desorption of ε-poly-L-lysine from the droplets surface and a charge reduction. Both situations might have derived to a poor electrostatic and steric stabilisation of the system, so attractive forces between droplets would have contributed on aggregation processes. The presence of a cream layer (top) and a serum layer (bottom) in tertiary emulsions with NaCl concentration over 0.3 M after 24 h treatment could be related to droplet aggregation as well as their low viscosity (data not shown). Subsequently, β-carotene content significantly decreased to values under 10% at NaCl concentrations over 0.3 M, which might be related to instability processes observed under these conditions (Fig. 6C). Gravitational separation of emulsions can be reduced by increasing the viscosity of the continuous phase, reducing movement of oil droplets (Zinoviadou, Scholten, Moschakis, & Biliaderis, 2012).

3.5. Lipid digestibility and β-carotene bioaccessibility

The final number of free fatty acids released was higher as the number of the layers in the emulsion increased: primary emulsion (34.61%), secondary emulsion (61.03%) and tertiary emulsion (83.79%) (Fig. 7). Although ε-poly-L-lysine might inhibit lipid digestion (Kido et al., 2003), the low amount of this polyelectrolyte present in tertiary emulsion (0.18% w/w) might not have been enough to affect the lipid digestion negatively. The acid pH conditions from the stomach phase (≈2.5) would have weakened the electrostatic attraction between ε-poly-L-lysine and alginate, third and second layer of the tertiary emulsion, respectively (Section 3.4.2). In addition, bile salts in combination with chymotrypsin and trypsin, would have displaced lactoferrin from the droplets interface during intestinal phase (Pilosof, 2017), facilitating lipid digestion.

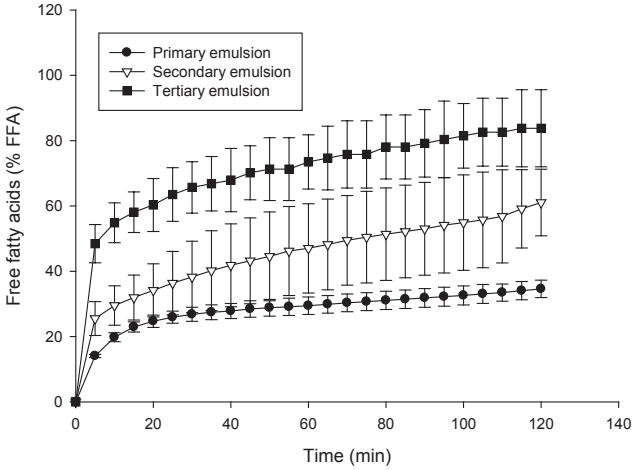

Fig. 7. Free fatty acids release (%FFA) from primary, secondary and tertiary emulsions during intestinal phase.

Alternatively, dilution of emulsions with simulated gastric fluids and the continuous agitation during this stage might have produced a redistribution of droplets and a reduction of droplet aggregation, contributing on physical stability of tertiary emulsion in stomach (Golding et al., 2011). Furthermore, ε-poly-L-lysine is able to inhibit the activity of pepsin at low pH conditions (Lawton & Mekras, 1985). Consequently, it would be proposed that tertiary emulsions presented a high physical stability under stomach conditions, and therefore their particle size would have remained small when entering within the intestinal phase. When these emulsions reached the intestine (neutral pH), the different layers would have been completely detached from the surface droplets. Acevedo-Fani, Salvia-Trujillo, Soliva-Fortuny, and Martín-Belloso (2015) reported a reduction of the surface ζ-potential of multilayer systems as the amount of adsorbed material in the layer diminishes, because there are less ionizable moieties that contribute on ζ-potential. Consequently, a less negative ζ-potential under neutral pH conditions (Section 3.4.2) could be associated with a decrease in thickness of the layer covering oil droplets, suggesting desorption of the different layers. Combination of the small particle size of tertiary emulsion when reaching the small intestine, together with the layer's desorption from oil droplets, would have facilitated the lipase action and therefore, lipid digestion of tertiary emulsion.

Indeed, β-carotene bioaccessibility significantly increased as emulsions had more interfacial layers, rising from 30.24% and 35.26% (primary and secondary emulsions, respectively) to 70.1% for tertiary emulsions. These results suggested that bioaccessibility might be associated with lipid digestibility, showing a higher β-carotene bioaccessibility as the free fatty acids release increased. The high β-carotene bioaccessibility observed for tertiary emulsions suggest that most of the β-carotene released from the lipid phase was solubilised within the mixed micelles formed during the intestinal digestion. Compared to other studies (Pinheiro, Coimbra, & Vicente, 2016; Silva et al., 2018; Tokle et al., 2013; Zhang et al., 2015), our bioaccessibility results were substantially greater. For instance, Tokle et al. (2013) obtained a β-carotene bioaccessibility below 2% for a multi-layer emulsion containing a similar amount of oil (2%) but with different emulsifiers (lactoferrin/β-lactoglobulin/lactoferrin). Low bioaccessibility results were attributed to the interaction between lactoferrin and β-carotene, remaining β-carotene entrapped in the sediment phase and not being solubilised within mixed micelles. Furthermore, studies about nanoemulsions (single-layered emulsions) have reported bioaccessibility results between 1.5 and 35% (Feng et al., 2017; Gasa-Falcon, Odriozola-Serrano, Oms-Oliu, & Martín-Belloso, 2019), suggesting that higher number of layers covering droplets (multi-layered emulsions) would improve bioaccessibility of encapsulated compounds. In order to

explain the high β-carotene bioaccessibility obtained in this study, a combination of different hypothesis was proposed. First, lipid digestibility as well as bioaccessibility might have been favoured because the presence of lipid content in tertiary emulsions was not in excess. Both lipase-to-lipid and bile-to-lipid ratio was high and thus, enough mixed micelles to incorporate the released β-carotene would have been generated. Second, antioxidant effects from lactoferrin (Huang, Satué-Gracia, Frankel, & German, 1999), alginate (Xue, Yu, Hirata, Terao, & Lin, 1998) and ε-poly-L-lysine (Scheffler, Wang, Huang, Gonzalez, & Yao, 2010) molecules would have protected β-carotene from degradation along the *in vitro* simulated gastrointestinal tract, but mostly from the acidic environment in stomach phase. Third, desorption of ε-poly-L-lysine, alginate and lactoferrin from droplets surface under neutral conditions, could have facilitated the access of lipase to the oil droplets. Bioaccessibility of lipophilic compounds such as β-carotene primarily depends on the amount of released compound from the lipid phase during digestion. Thus, the relatively high free fatty acids liberated (70%), might have indicated that lipids from tertiary emulsion were hydrolysed and that β-carotene was released from oil droplets.

4. Conclusions

A tertiary emulsion (lactoferrin/alginate/ε-poly-L-lysine) containing a high oil concentration (2.5% w/w) was successfully developed. Selecting the optimal polyelectrolyte concentration of each layer was essential in order to avoid destabilization processes. Tertiary emulsions of this work would be most suitably used in neutral foods and beverages, in thermal processes below 60 °C as well as food products without salt due to its high physical stability. In addition, β-carotene content of around 30% was maintained under these conditions. Finally, although the numerous layers of polyelectrolyte covering the oil droplets, tertiary emulsions presented a greater lipid digestibility and β-carotene bioaccessibility compared to primary and secondary emulsions, suggesting that multilayered emulsions might be used as a new approach to enhance delivery of encapsulated lipophilic compounds. An in-depth study is needed in order to evaluate the application of this tertiary emulsion within food products.

Acknowledgements

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